

## Determination of the Activity of Diclazuril Against *Sarcocystis neurona* and *Sarcocystis falcatula* in Cell Cultures

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**ABSTRACT:** Diclazuril is a benzenacetone nitril anticoccidial that has excellent activity against the extraintestinal stages of *Toxoplasma gondii* and *Neospora caninum*. It also is highly active against intestinal coccidia of poultry. The present study examined the efficacy of diclazuril in inhibiting merozoite production of *Sarcocystis neurona* and *Sarcocystis falcatula* in bovine turbinate cell cultures. Diclazuril inhibited merozoite production by more than 80% in cultures of *S. neurona* or *S. falcatula* treated with 0.1 ng/ml diclazuril and greater than 95% inhibition of merozoite production was observed when infected cultures were treated with 1.0 ng/ml diclazuril. Diclazuril may have promise as a therapeutic agent in the treatment of *S. neurona*-induced equine protozoal myeloencephalitis in horses and *S. falcatula* infections in birds.

Equine protozoal myeloencephalitis (EPM) is a neurologic syndrome in horses caused primarily by infection with *Sarcocystis neurona*. The condition has been recognized for over 20 yr (Beech, 1974; Beech and Dodd, 1974; Cusick et al., 1974; Dubey et al., 1974), and EPM is the most important protozoal disease of horses (MacKay et al., 1992). Recent serological surveys indicate that 45–53% of horses have antibodies to *S. neurona* (Bentz et al., 1997; Blythe et al., 1997; Saville et al., 1997).

*Sarcocystis falcatula* is highly pathogenic for several species of pet or exotic birds. Clubb and Frenkel (1992) reported histologically confirmed acute sarcocystosis in 12 species of psittacine birds raised in outdoor aviaries on 2 farms in Florida. Hillyer et al. (1991) described deaths in 21 species (37 birds total) of Old World psittacines at the San Diego Zoo in California due to sarcocystosis. Both reports indicated that *S. falcatula* was the cause of the observed deaths.

Studies using the small subunit ribosomal RNA sequences indicated that *S. neurona* and *S. falcatula* are the same parasite (Dame et al., 1995; Fenger et al., 1995). However, recently it has been demonstrated that the 2 species are distinct (Marsh et al., 1997; Dubey and Lindsay, 1998; Tanhauser et al., 1999) but that both use the opossum, *Didelphis virginiana*, as a definitive host (Dubey and Lindsay, 1998).

Diclazuril is a benzenacetone nitril anticoccidial that has excellent activity against *Toxoplasma gondii* and *Neospora caninum* in vitro (Lindsay and Blagburn, 1994; Lindsay et al., 1994; Lindsay, Rippey, Toivio-Kinnucan, and Blagburn, 1995). Diclazuril prevents toxoplasmosis in mice (Lindsay and Blagburn, 1994) and is highly effective in treating acute toxoplasmosis in mice (Lindsay, Rippey, and Blagburn, 1995). Diclazuril is fed at 1 part per million (ppm) to prevent intestinal coccidiosis caused by *Eimeria tenella*, *Eimeria acervulina*, *Eimeria mitis*, *Eimeria brunetti*, *Eimeria necatrix*, and *Eimeria maxima* in chickens (McDougald et al., 1990) and 1 ppm to prevent coccidiosis caused by *Eimeria adenoides*, *Eimeria gallopavonis*, and *Eimeria meleagridis* in turkeys (Vanparijs et al., 1989).

*Sarcocystis neurona* merozoites (SN6 strain, isolated from a horse with EPM; Dubey et al., 1999) and *S. falcatula* (Cornell strain, isolated from the lungs of budgerigars, *Melopsittacus undulatus*, fed sporocysts from an opossum; Lindsay et al., 1999) merozoites were grown and maintained in bovine turbinate (BT cells, ATTC CRL 1390) as described previously (Lindsay et al., 1999). The BT cells were grown to confluence in 25-cm<sup>2</sup> plastic cell culture flasks in growth media that consisted of 10% (v/v) fetal bovine serum (FBS) in RPMI-1640 medium supplemented with 100 U penicillin G/ml and 100 mg streptomycin/ml. Cell cultures were maintained in growth medium in which the FBS content is lowered to 2%. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air.

Merozoites were grown and maintained in BT cells. For quantitative studies, merozoites were harvested from infected cell cultures by removing the medium and replacing it with Hanks' balanced salt solution without calcium and magnesium. The BT cells were then removed from the plastic growth surface by use of a cell scraper. This cell mixture was passed through a 27-gauge needle attached to a 10-ml syringe to rupture host cells. The suspension was then filtered through a sterile 3-μm filter to remove cellular debris. The numbers of merozoites in the filtrate was determined using a hemacytometer. The final volume of suspension was adjusted so  $5 \times 10^5$  merozoites were present for inoculation.

For general maintenance of merozoites, monolayers were examined with an inverted microscope for the development of lesions (areas devoid of host cells caused by parasite replication) in the monolayer or the presence of many extracellular merozoites. Once lesions were observed or many extracellular parasites were present, the monolayer was scraped with the tip of a 5-ml pipette and 1–3 drops of the merozoite-containing fluid were transferred to 2 flasks of BT cells. Merozoites of *S. neurona* were passaged in this manner every 3–7 days and merozoites of *S. falcatula* were passaged every 7–14 days.

The activity of diclazuril (lot PFA101; Pitman-Moore, Inc., Kansas City, Kansas) was determined in a merozoite production (MP) assay (Lindsay and Blagburn, 1994). Diclazuril was dissolved in dimethylsulfoxide (DMSO) and to make a stock solution of 1 mg/ml. Dilutions were made from this stock solution and the highest concentration of DMSO in any solution was 0.01% (v/v). Previous studies with several antimicrobial agents have shown that this amount of DMSO has no effect on BT cells or *S. neurona* merozoite development (Lindsay and Dubey, 1999); therefore DMSO was not used in the control media. Cell monolayers were inoculated with  $5 \times 10^5$  merozoites of *S. neurona* or *S. falcatula*. Two hours after inoculation, the me-

TABLE I. Percent reduction in merozoite production in flasks of bovine turbinate cells infected with *Sarcocystis neurona* and *Sarcocystis falcatula* and treated with various concentrations of diclazuril.

	Concentration of diclazuril (ng/ml)	% Reduc- tion*	Tukey's test†
<i>S. neurona</i> , experiment 1	None	NA	a
	0.1	86.7	b
	1.0	93.9	b, c
	10.0	96.1	c
	100.0	96.1	c
<i>S. neurona</i> , experiment 2	None	NA	a
	0.001	0	a
	0.01	36.8	b
	0.1	86.0	c
<i>S. falcatula</i> , experiment 3	None	NA	a
	0.01	34.1	a
	0.1	84.8	b
	1.0	97.0	c
	10.0	97.2	c

\* Negative % reductions are expressed as 0.

† Concentrations with different letters within an experiment are significantly different ( $P < 0.05$ ).

medium was removed and replaced with maintenance medium containing diclazuril at various ng/ml concentrations (Table I; Fig. 1). Control flasks received maintenance medium without diclazuril. Four flasks were used per diclazuril treatment dose. The merozoite production assay was conducted after 10 days of treatment of *S. neurona*-infected cultures or 13 days of treatment of *S. falcatula*. These times were chosen so that 3 asexual generations of each parasite would be exposed to the diclazuril treatment. The medium was removed from the cultures, the volume was recorded, and the numbers of merozoites determined by counting in a hemacytometer. The total number of merozoites present in each flask was determined by multiplying the volume of medium by the numbers of merozoites (mean of 16 counts/treatment [4 counts/flask])/ml of medium. The percentage reduction in merozoites due to treatment was calculated by subtracting the mean treated values from the mean control value, dividing this numerator by the mean control value, and multiplying the product by 100.

The following procedure was used to determine if diclazuril treatments killed developing stages of *S. neurona* or *S. falcatula*. After the medium was collected for the MP assay, the cell monolayer was rinsed twice with maintenance medium to wash off any residual diclazuril, and 5 ml of maintenance medium was added to the flask. The flasks were then examined for 30 days for renewed growth of parasites, or monolayer destruction, or both. Two experiments (experiments 1 and 2) were done with *S. neurona* and 1 experiment was done with *S. falcatula* (experiment 3).

For experiments 1 and 3 counts were log transformed to stabilize variances before analysis and then back-transformed for presentation. The MIXED procedure of SAS (SAS version 6.12, SAS Institute Inc., Cary, North Carolina 27513) was used to estimate variance components, perform analysis of variance, and calculate 95% confidence intervals. Tukey's HSD was used to compare means.

Analysis of variance components indicated that no significant

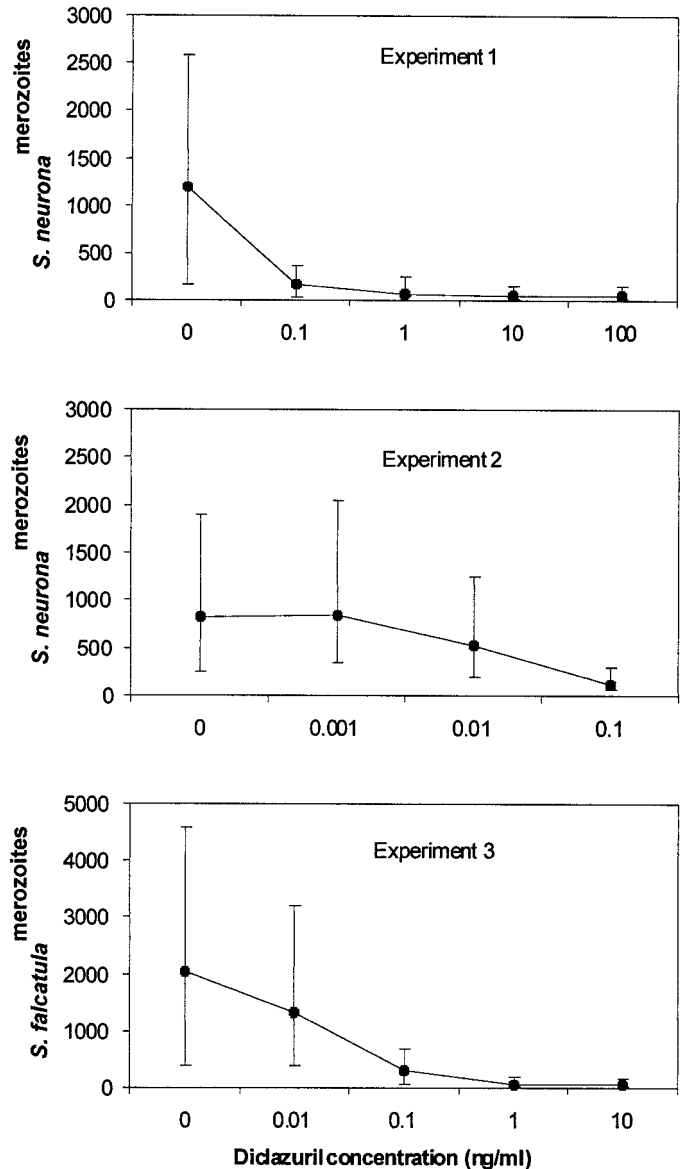


FIGURE 1. Mean merozoite counts/flask of *Sarcocystis neurona* (experiments 1 and 2) and *Sarcocystis falcatula* (experiment 3) in bovine turbinate cell cultures treated with ng/ml concentrations of diclazuril. Bar = 95% confidence intervals.

differences ( $P > 0.05$ ) were present in total merozoite counts between flasks undergoing the same diclazuril treatment within an experiment. In experiments 1 and 2 BT cells in flasks infected with *S. neurona* and not treated with diclazuril had approximately 40–50% monolayer destruction when the MP assay was conducted. Bovine turbinate cells in flasks infected with *S. neurona* and treated with  $\geq 1.0$  ng/ml diclazuril in experiments 1 and 2 and had no visible monolayer destruction, those treated with 0.1 ng/ml had 5% monolayer destruction, and those treated with  $< 0.1$  ng had 40–50% monolayer destruction when the MP assay was conducted. In experiment 3, BT cells in flasks infected with *S. falcatula* and treated or not treated with diclazuril exhibited the same concentration-dependent pattern of monolayer destruction as observed for *S. neurona*-infected cultures when the MP assay was conducted.

Diclazuril had good activity against both parasites (Table I; Fig. 1). Concentrations of 1.0 ng/ml inhibited merozoite production by greater than 95% of the controls in both species. Diclazuril inhibited development but did not completely inhibit merozoite production at the levels tested. Renewed multiplication occurred in both *S. neurona*- and *S. falcatula*-treated flasks when the diclazuril-containing medium was removed.

Cell culture studies indicate that the efficacy of diclazuril against *T. gondii* tachyzoites (Lindsay and Blagburn, 1994) and *N. caninum* tachyzoites (Lindsay et al., 1994) is similar to that observed for against *S. neurona* in the present study. Diclazuril will prevent death in 80 and 100% of mice due to RH strain *T. gondii* if given orally once daily at 1.0 or 10 mg/kg on 1 day prior to infection and then daily for 10 days (Lindsay and Blagburn, 1994). Diclazuril will prevent death in 90% of mice with acute RH strain toxoplasmosis when given orally at 10 mg/kg once daily for 10 days beginning on day 6 when mice are clinically ill (Lindsay, Rippey, and Blagburn, 1995). The activity of diclazuril against acute toxoplasmosis in mice is also augmented when combined with pyrimethamine (Lindsay, Rippey, and Blagburn, 1995). These findings suggest that the activity observed in vitro should translate to activity in the equine host.

Diclazuril is fed to turkeys and chickens at 1 ppm to prevent intestinal coccidiosis (Vanparijs et al., 1989; McDougald et al., 1990). Experimental studies also indicate that when fed at 2–4 ppm diclazuril will prevent coccidiosis in pheasants (Vanparijs et al., 1990) and partridges (Vanparijs et al., 1991). It is likely that feeding diclazuril at 1–4 ppm to psittacines who are at risk would also prevent acute *S. falcatula* infections.

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